

PATENT
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APPLICATION FOR UNITED STATES
LETTERS PATENT
for
METHOD FOR THE DEVELOPMENT OF AN HIV VACCINE
by
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<u>EXPRESS MAIL MAILING LABEL</u>	
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1.0 BACKGROUND OF THE INVENTION

This application is a continuation-in-part of and claims priority to International Patent Application No. PCT/US99/03217 filed February 12, 1999, designating the United States, which claims priority to U.S. Provisional Patent Application Serial No. 60/074,646, filed February 13, 1998. This application is also a continuation-in-part of and claims priority to pending U.S. Patent Application Serial No. 09/249,391 filed February 12, 1999, which claims priority to U.S. Provisional Patent Application Serial No. 60/074,646 filed February 13, 1998.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of virology, immunology, disease treatment and prevention. More particularly, it concerns HIV particles with inactivated reverse transcriptase, methods of inactivation, and the use of such particles to prepare components of HIV and to elicit effective immunological responses to HIV.

These immune responses are useful in producing diagnostic reagents, assays, and kits for the diagnosis of HIV and related retroviral disease, providing protection from an HIV challenge, and assisting an HIV-infected individual in controlling the replication of the virus. Methods of inactivation are useful for preventing disease through decreasing the risk of infection associated with exposure to HIV infected tissues and materials.

1.2 DESCRIPTION OF RELATED ART

1.2.1 HUMAN IMMUNODEFICIENCY VIRUS

Human Immunodeficiency Virus-1 (HIV-1) infection has been reported throughout the world in both developed and developing countries. HIV-2 infection is found predominately in West Africa, Portugal, and Brazil. It is estimated that as of 1990 there were between 800,000 and 1.3 million individuals in the United States that were infected with HIV. An important obstacle to developing a vaccine against HIV is that the mechanism of immunity to HIV infection is ill-understood. Not all of those infected individuals will develop acquired immunodeficiency syndrome (AIDS). Indeed recent

reports have suggested that there may be certain individuals that are resistant to HIV-1 infection.

5 The HIV viruses are members of the Retroviridae family and, more particularly, are classified within the Lentivirinae subfamily. Like nearly all other viruses, the replication cycles of members of the Retroviridae family, commonly known as the retroviruses, include attachment to specific cell receptors, entry into cells, synthesis of proteins and nucleic acids, assembly of progeny virus particles (virions), and release of progeny viruses from the cells. A unique aspect of retrovirus replication is the
10 conversion of the single-stranded RNA genome into a double-stranded DNA molecule that must integrate into the genome of the host cell prior to the synthesis of viral proteins and nucleic acids.

Retrovirus virions are enveloped and contain two copies of the genome. The
15 conversion of the genomic RNA into DNA is provided by the viral protein reverse transcriptase (RT). This protein is bound to the RNA genome within the virion, and its enzymatic conversion of the genome to DNA is believed to take place after viral entry into the host cell. However, recent evidence suggests that the conversion process may initiate in the virion particles themselves, known as endogenous reverse transcription (ERT), and that ERT may be important in increasing the infectivity of the virus in sexual
20 transmission (Zhang *et al.*, 1993, 1996).

Because of the requirement for reverse transcription in the viral replication cycle, compounds that interfere with RT activity have been utilized as anti-HIV therapeutic
25 agents. Many of these compounds, including 3'-azido-2', 3'-dideoxythymidine (AZT), are nucleoside analogs that, upon activation by host cell kinases, are competitive inhibitors of reverse transcriptase (Furman *et al.*, 1986). Other anti-RT compounds are nonnucleoside inhibitors (NNI), hydrophobic compounds that do not require cellular modification for antiviral activity. Examples of such compounds include nevirapine
30 (Grob *et al.*, 1992; Merluzzi *et al.*, 1990), the pyridinones (Carroll *et al.*, 1993; Goldman

et al., 1991), and the carboxanilides (Bader et al., 1991; Balzarini et al., 1995, 1996). The nevirapine analog 9-azido-5,6-dihydro-11-ethyl-6-methyl-11H-pyrido[2,3-b][1,5]benzodiazepin-5-one (9-AN) and the carboxanilide analog N-[4-chloro-3-(3-methyl-2-butenyloxy)phenyl]-2-methyl-3-furanocarbothiamide (UC781™) have been shown to be potent inhibitors of RT. In respect of the 9-AN, the exposure of a mixture of this compound and RT to UV-irradiation has been particularly effective in inhibiting RT. From the series of carboxanilides compounds, UC781™ has been found to be particularly effective (Barnard et al., 1997). The addition of a photoreactive label to UC781™ should increase further its ability to inactivate HIV RT, when a mixture of UC781™ and RT is exposed to UV-irradiation. The irradiation of a mixture of a photolabeled NNI of RT and RT is a type of photoinactivation.

The binding affinity and inhibitory effect of UC781™ is so high that the compound was able to eliminate HIV infectivity following short exposure of the isolated virus to UC781™ without the need for photoinactivation (Borkow et al., 1997). Further, this compound was shown to inhibit ERT in HIV virions and, when provided to HIV infected cells, caused the production of noninfectious nascent virus (Borkow et al., 1997). Therefore, it appears that UC781™ is a particularly powerful inactivator of HIV. Although UC781™ has been proposed for use in retrovirucidal formulations (Borkow et al., 1997), use as a photoinactivator of HIV for the purpose of producing an non-infectious virus particle useful as an immunogen is absent from the prior art.

Non-infectious, virus-like particles have previously been produced via manipulations of the viral genome. For example, U.S. Patent 6,080,408, incorporated herein by reference, discloses non-infectious virus-like particles wherein RT has been made inactive by virtue of deletions and sequence changes in the viral genome. Rovinski also discloses other methods of making non-infectious, virus-like particles, (or pseudovirions), that involve alterations to other genes of the HIV genome, expression of a subset of HIV virion components, and heat-inactivation of sera from HIV infected

individuals. U.S. Patent 6,017,543 discloses methods using formalin, psoralen, beta-propiolactone, alone and in combination, as well as exposure to gamma radiation.

1.2.2 IMMUNE RESPONSE TO HIV

5 The immune response to HIV is composed of an initial cell mediated immune response followed by the subsequent development of neutralizing antibodies. Within weeks of infection, virus titers in the blood fall coincident with the induction of anti-HIV cellular and humoral immune responses. The fall in viremia correlates well with the appearance of anti-HIV major histocompatibility complex (MHC) class I-restricted CD8⁺ cytotoxic T cells (Haynes *et al.*, 1996). Recent evidence has shown a strong correlation of anti-HIV CD4⁺ T cell responses and reduced viral loads (Rosenberg *et al.*, 1997). Therefore, the presentation of HIV antigens in the context of MHC class II molecules to CD4⁺ T cells may be the key aspect of the control of the HIV infection.

15 Rosenberg *et al.* (1997) suggest that in HIV-1 infection, HIV-specific CD4⁺ cells may be selectively eliminated. This may be due to the activation of these cells during high-level viremia, increasing their susceptibility to infection (Weissman *et al.*, 1996; Stanley *et al.*, 1996), or may be due to activation induced cell death during primary infection (Abbas, 1996). Nonetheless, increasing the virus-specific CD4⁺ T cell response without infecting, or destroying, the responding cells may be an effective means of controlling viral loads. Therefore, some existing HIV vaccines may be ineffective because they do not provide presentation of HIV peptides in the context of MHC class II by antigen presenting cells.

25 1.2.3 HIV VACCINES

Historically, viral vaccines have been enormously successful in the prevention of infection by a particular virus. Therefore, when HIV was first isolated, there was a great amount of optimism that an HIV vaccine would be developed quickly. However, this optimism quickly faded because a number of unforeseen problems emerged. A

discussion of the problems that an HIV vaccine must overcome is provided within Stott and Schild (1996) and is incorporated herein by reference.

5 First, HIV is a retrovirus, thus, during its growth cycle, proviral DNA is integrated
in the host genome. In this form the virus is effectively protected from the immune
response of the host and this feature of the virus suggests that effective vaccination must
ideally prevent the initial virus-cell interaction following transmission. Few, if any, of
the currently available successful viral vaccines against other infections achieve this level
of protection. Secondly, HIV specifically targets and destroys T-helper lymphocytes,
10 which form an essential component of the immune response. Thirdly, the virus is capable
of extremely rapid antigenic variation which permits escape of the virus from immune
responses. Fourthly, the majority of infections are acquired sexually via the genital or
rectal mucosae, and infections of this route are generally considered difficult to prevent
by vaccination. Finally, infection may be transmitted by virus-infected cells in which the
15 proviral DNA is integrated and viral antigens are not expressed. Such a cell would not be
recognized by immune responses to viral proteins and would therefore pass undetected.
Few data are available to indicate how significant this mode of transmission is in the
overall epidemiology of HIV-1. Nevertheless, it represents a potential route and one
which some authorities believe cannot be blocked by vaccination (Sabin, 1992).

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Types of HIV vaccines include inactivated virus vaccines, live attenuated virus
vaccines, virus subunit vaccines, synthetic particle vaccines, and naked DNA vaccines
and are reviewed in Stott and Schild (1996), Schultz (1996), and Johnston (1997).
Several of these vaccines are already in human trials.

25

The first evidence that vaccination against immunodeficiency viruses was feasible
came from early experiments using simple inactivated virus prevented the onset of
disease when vaccinated animals were subsequently challenged (Desrosiers *et al.*, 1989;
Sutjipto *et al.*, 1990). These results were confirmed and extended by Murphey-Corb
30 *et al.* (1989) who showed that most animals immunized with formalin-inactivated virus

were protected against infection with SIV. Similar results were subsequently obtained by several laboratories using virus-infected cells (Stott *et al.*, 1990) or partially purified virus, inactivated by aldehydes (Putkonen *et al.*, 1991, 1992; Johnson *et al.*, 1992a; Le Grand *et al.*, 1992), β -propiolactone (Stott *et al.*, 1990) detergent (Osterhaus *et al.*, 1992) or psoralin and UV light (Carlson *et al.*, 1990). Several different isolates of SIV or infectious molecular clones derived from them were used to prepare the vaccine and challenge viruses. A wide variety of adjuvants were also employed. On every occasion vaccinated macaques were protected against infection by intravenous challenge of between 10-50 MID₅₀ (50% monkey infectious doses). Infections virus could not be recovered from the blood or tissues of the protected animals even when they were followed for prolonged periods of over 1 year. Even more impressive was the failure to detect proviral DNA in the lymphocytes of protected animals, indicating that there had been no integration of the challenge virus (Stott *et al.*, 1990; Johnson *et al.*, 1992a). It was thus clear that inactivated virus vaccines induced a powerful protective response in macaques. Unfortunately, the protection induced by inactivated SIV in macaques was not reproduced in chimpanzees vaccinated with inactivated HIV and challenged with HIV-1 (Warren and Doltshahi, 1993).

1.2.4 PHOTOINACTIVATION OF HIV

Methods of photoinactivation of HIV are known in the art and have been the subject of at least three patents. U.S. Patent 5,041,078 describes the use of sapphyrins in the photodynamic inactivation of viruses, including HIV. U.S. Patents 5,516,629 and 5,593,823 describe the use of psoralens and ultra violet light to inactivate HIV. U.S. Patents 5,516,629 is incorporated herein by reference. Psoralens are naturally occurring compounds which have been used therapeutically for millennia in Asia and Africa. A psoralen binds to nucleic acid double helices by intercalation between base pairs. Upon absorption of UVA photons, the psoralen excited state has been shown to react with a thymine or uracil double bond and covalently attach to both strands of a nucleic acid helix. The crosslinking reaction is specific for a thymine (DNA) or uracil (RNA) base and will proceed only if the psoralen is intercalated in a site containing thymine or uracil.

The initial photoadduct can absorb a second UVA photon and react with a second thymine or uracil on the opposing strand of the double helix to crosslink the two strands of the double helix.

5 Lethal damage to a cell or virus occurs when a psoralen intercalated into a nucleic acid duplex in sites containing two thymines (or uracils) on opposing strands sequentially absorb 2 UVA photons. This is an inefficient process because two low probability events are required, the localization of the psoralen into sites with two thymines (or uracils) present and its sequential absorption of 2 UVA photons.

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Attempts to inactivate viruses using photosensitizers and light have also been developed using some non-psoralen photosensitizers. The photosensitizers that have been employed are typically dyes. Examples include dihematoporphyrin ether (DHE), Merocyanine 540 (MC540) and methylene blue.

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Carlson *et al.* (1990) has shown that a psoralen-inactivated whole SIV (the Simian counterpart of HIV) vaccine can protect against low challenge doses of SIV and prevent early death in those monkeys that do become infected, suggesting that inactivated HIV may be an effective vaccine in humans. However, because photoinactivation using psoralens is dependent on two rare events, a more efficient method of inactivation is preferable to decrease the likelihood of live virus within a sample. Furthermore, these methods alter the antigenic conformation of HIV affecting the production of an effective immunological response.

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25 1.3 DEFICIENCIES IN THE PRIOR ART

Due to previous successes in preventing viral diseases using subunit, live-attenuated viral, and inactivated viral vaccines, the scientific community was initially optimistic that a vaccine would be developed to prevent the spread of HIV. However, early optimism soon diminished because of repeated failures in the development of an effective vaccine.

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Vaccines based on subunit immunogens, although extremely safe, are limited in the breadth of antigens that are presented to the immune system because only one or a few of the viral proteins are utilized as immunogens. This may limit the likelihood of cross protection between clades of HIV. Also, the production of vaccines based upon subunit immunogens requires the molecular manipulation of the viral proteins into cloning or expression vectors, perhaps leading to increased production time and costs.

Live-attenuated HIV vaccines typically utilize whole, or nearly whole virions as immunogens. Production of these vaccines may require molecular manipulations of the HIV genome in their production, although spontaneous attenuated viruses may occur naturally. Attenuated HIV vaccines have included deletions in the *nef* region of the virus. Mutant-*nef* SIV vaccines showed initial promise in primates, however, it was quickly shown that these vaccines were capable of causing disease in newborn animals. Furthermore, recent evidence suggests that these vaccines are capable of causing full-blown AIDS in adult monkeys (Cohen, 1997). Therefore, the lack of an efficient understanding of HIV and its pathogenesis makes the use of attenuated viruses a risky endeavor.

Inactivated viral vaccines provide a larger complement of immunogens presented to the immune system, and, therefore, provide a greater amount of protection from HIV and are more likely to provide protection across HIV clades. Furthermore, the inactivated viral vaccines do not require molecular manipulation of HIV and can be made to essentially any strain. Because inactivation of the virus is readily shown in *in vitro* and animal models, the inactivated HIV vaccines are able to be tested in a timely manner to determine the effectiveness of inactivation. Attenuated viruses may take years to determine the effectiveness of the vaccine.

To be safe to administer to humans, efficient methods of inactivation of HIV are required for vaccine production. Methods known for the inactivation include the use of

aldehydes, β -propiolactone, psoralin and UV light, and others including detergents. Many of these methods alter the conformation of the virus thereby altering the specificity of the immune response to the virus. Even gamma radiation may require the additional use of protective compounds to help preserve viral protein integrity, as disclosed in U.S. Patent 6,017,543. Photoinactivation of HIV using psoralin and UV light does not alter the conformation of the virus, but it is an inefficient method of inactivation. Therefore, more efficient methods of inactivation that do not affect the conformation of the viral immunogen would be ideal for use in the production of an HIV vaccine.

2.0 SUMMARY

The prior art is devoid of HIV vaccines, HIV immunogens, and HIV viral particles created using an efficient method of inactivating HIV without causing deformation of the viral particle. The viral particles of the present invention are produced using efficient methods of inactivation that do not alter the conformation of the virus to the same extent as other inactivation methods. Moreover, the seriousness of the risk of HIV infection in those working with the virus or infected hosts or tissues dictates that non-infective virions must be inactivated in a highly efficient manner. Such non-infective virions are provided by the present invention. The photoinactivated viral particles themselves are of use in the preparation of isolated viral components. The methods of the present invention provide for the inactivation of HIV as a step in reducing the risk of HIV infection to those who work with the virus in various stages of HIV research, including virus particle isolation and analysis.

The viral particles of the present invention mimic infectious HIV particles but do not cause infection (*i.e.*, establishment of a perpetuation of HIV within the recipient host due to incorporation of HIV into the genome of cells within that host) and may be used as immunogens in a vaccine or in the development of effective treatments of HIV infection. Non-infectious HIV particles are useful not only in vaccination, but also in diagnosis, manipulation, and development of treatments for HIV infection. HIV particles rendered non-infective by photoinactivation may be used as an immunogen for making antibodies,

or screening for antibody-like binding compounds that recognize and bind to native HIV particles. These antibodies and other compounds provide means for accurate identification, quantification, and monitoring of HIV particles and infection. These uses and techniques are particularly disclosed in U.S. Patents 6,080,408 and 5,919,458, both of which are incorporated herein by reference.

The use of an azido dipyrroliazepinone and an azido thiocarboxanilide (azido UC781™), azido-labeled compounds shown to bind and inactivate RT, permits the generation of non-infectious particles of HIV by inactivating the HIV RT upon exposure of infectious particles to either compound followed by irradiation with ultraviolet light. The effective inactivation of HIV RT by the methods described herein allows the production of non-infectious particles of HIV. These non-infectious particles of HIV have the capacity of eliciting an effective cell mediated and antibody mediated immune response which is protective against infection by HIV. The inactivated HIV particles of the present invention preserve the antigenic composition of infectious wild-type HIV particles and thereby facilitate the dendritic cell-mediated processing and presentation of HIV particle-derived antigens to T cells. The inactivated HIV particles are effective immunogens.

The application of this methodology to different strains of HIV may allow the production of a polyvalent vaccine (NIIPAV or NON-Infectious Immunogenic Polyvalent AIDS Vaccine). The inactivated HIV particles of the present invention upon binding to CD4 receptors will expose epitopes which may elicit broad immunogenic responses capable of inhibiting the infectivity of diverse types of HIV from different clades. Thus the exposure of the immune system to a single type of inactivated particle of the present invention may protect against infection by multiple types of HIV.

One aspect of the present invention is a composition comprising an HIV particle in which the RT is inactivated. The composition may further comprise a pharmaceutically-acceptable excipient. The present invention contemplates that the HIV

particle may be any type, subtype or isotype of HIV. In a preferred embodiment, the HIV particle is a wild type HIV particle. In one embodiment the HIV particle is HIV 1. The HIV 1 particle may be Group M or Group O. In different embodiments the Group M HIV 1 may be clade A, clade B, clade C, clade D, clade E, clade F, clade G, clade H, or
5 clade I. In a preferred embodiment of the invention, the Group M particle is a clade B particle.

In one embodiment the RT is inactivated by one or more compounds that binds the RT and then irradiating bound RT with UV light. In a preferred embodiment the UV
10 light is that emitted by a GE 275 W sun lamp. However, it is contemplated that any light that causes the reaction of the compound with RT may be used. In one embodiment of the compound that binds to RT is an azido labeled compound. Essentially any azido-labeled compound that binds to HIV RT and can penetrate the HIV particle so as to associate with RT may be used. In a preferred embodiments the azido-labeled compound
15 is azido dipyrodiazepinona or azido-UC781™. In other embodiments the azido-labeled compound is the azido derivative of 9-AN, UC38, UC84, UC10, UC82, UC040, HBY 097, calanolide A, or U-88204E. In one embodiment the inactivation of RT comprises contacting said HIV particle with an effective amount of the azido labeled compound.

20 Another embodiment of the invention comprises HIV particles inactivated by contact with a compound capable of inactivating RT and irradiation with UV light. In preferred embodiments the compound inactivating RT is an azido-labeled compound. In other, preferred embodiments, the azido-labeled compound is azido dipyrodiazepinona or azido-UC781™. In other embodiments the azido-labeled compound is the azido
25 derivative of 9-AN, UC38, UC84, UC10, UC82, UC040, HBY 097, calanolide A, or U-88204E. The present invention contemplates that the HIV particles may be any type, subtype or isotype of HIV. In a preferred embodiment, the HIV particle is a wild type HIV particle. In one embodiment the HIV particle is HIV 1. The HIV 1 particle may be Group M or Group O. In different embodiments the Group M HIV 1 may be clade A,

clade B, clade C, clade D, clade E, clade F, clade G, clade H, or clade I. In a preferred embodiment of the invention, the Group M particle is a clade B particle.

Another aspect of the present invention is a method of invoking an immune response in an animal by administering a composition comprising a pharmaceutically-acceptable excipient and an HIV particle in which the RT is inactivated. The immune response may be a humoral response, a cellular response or both a humoral and cellular response. The cellular response may be a CD8+ T cell response, a CD4+ T cell, or both a CD8+ T cell and a CD4+ T cell response.

The animal in which the immune response is invoked may be a mammal. In preferred embodiments the mammal may be a human, a PBL-SCID mouse, or a SCID-hu mouse. The animal in which the immune response is invoked may be either HIV positive or HIV negative.

Another aspect of the present invention is a method of delaying the onset of AIDS in an animal exposed to infectious HIV by administering to the animal an inoculation of a pharmaceutically acceptable excipient and an HIV particle in which the RT is inactivated. The animal may be a mammal and in preferred embodiments the mammal is a human, a PBL-SCID mouse, or a SCID-hu mouse. The animal may be either HIV negative or HIV positive at the time of the administration of the inoculation.

Another aspect of the present invention is a method of making an HIV particle containing an inactivated RT comprising contacting a compound capable of inactivating RT with an HIV particle such that the compound binds to the RT and then irradiating the HIV particle. In one embodiment the HIV particle is HIV-1. The HIV-1 particle may be Group M or Group O. In different embodiments the Group M HIV-1 may be clade A, clade B, clade C, clade D, clade E, clade F, clade G, clade H, or clade I. In a preferred embodiment of the invention, the Group M particle is a clade B particle.

In one embodiment of the compound capable of inactivating RT is an azido labeled compound. Essentially any azido-labeled compound that binds to HIV RT and can penetrate the HIV particle so as to associate with RT may be used. In preferred embodiments the azido-labeled compound is azido dipyrroldiazepinona or azido-UC781™.

5 In other embodiments the azido-labeled compound is the azido derivative of 9-AN, UC38, UC84, UC10, UC82, UC040, HBY 097, calanolide A, or U-88204E.

Another aspect of the present invention is a method of preparing a composition comprising making an HIV particle containing an inactivated RT by contacting a

10 compound capable of inactivating RT with an HIV particle such that the compound binds to the RT, irradiating the HIV particle, and then combining the HIV particle with the inactivated RT with a pharmaceutically acceptable excipient.

A further aspect of the present invention is a method of determining the presence

15 of retroviral antigens in a sample, comprising immunizing a host with one or more compositions as provided herein to produce HIV-specific antibodies, contacting these antibodies with the sample so that complexes of sample antigens and antibodies are produced, and determining the production, presence, or abundance of these complexes.

20 In another aspect, the invention features a diagnostic kit for detecting retroviral antigens in a sample. The kit comprises one or more HIV-specific antibodies as provided herein, means for contacting at least one of the specific antibodies with the sample to produce complexes of the antibody or antibodies and antigen or antigens, and means for determining the production, presence, or abundance of such complexes.

25 In yet a further aspect, the invention provides for inactivated viral particles that are of use in the preparation of isolated viral components. including, but not limited to, native capsid proteins, glycoproteins, and phosphoproteins. Additionally, the methods of the present invention provide for the inactivation of HIV as a step in reducing the risk of

30 HIV infection to those who work with the virus in various stages of HIV research,

including virus particle isolation and analysis. Viral particles that are inactivated can be stored and employed as a source of components for studies on HIV components, without the risks incumbent in storing active viruses. Therefore, the inactivated viral particles have a utility as a reagent that can be employed in the study of HIV. Likewise, the methods of the invention have utility in producing safe sources of HIV components.

3.0 BRIEF DESCRIPTION OF THE DRAWINGS

The following drawing forms part of the present specification and is included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Simplified phylogenetic tree of HIV-1. Group M denotes the major group of HIV-1; group O refers to outliers or outgroup. Sequence data from the *env* gene C2V3 region of selective isolates of subtypes A through H were compared to construct the phylogenetic tree. A ninth subtype, I, has been reported recently but is not shown because the isolate was characterized using a different segment of the *env* gene. The horizontal branch lengths represent approximate relative genetic distances. (From Hu D J, Dondero TJ, Rayfield MA, *et al.*, The emerging genetic diversity of HIV: The importance of global surveillance for diagnostics, research, and prevention. JAMA 275:210, 1996.)

4.0 DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention relates to a vaccine comprising RT-inactivated HIV for the purpose of eliciting a protective immune response in an animal. The present invention employs methods of inactivating RT. Further, the use of these inactivation methods for the purpose of producing a vaccine is novel.

There are well described methods for the isolation and culture of HIV. What is important to keep present in regard to the objectives of this invention is the fact that,

using a methodology that inactivates the HIV reverse transcriptase, non-infectious particles of HIV are obtained as immunogens capable of eliciting an effective immune response or the production of HIV-specific antibodies. Thus, although it makes sense to describe the method using specific strains of HIV and specific types of cells for the culture and testing of the lack of infectiousness of the generated particles, it should be kept in mind that this method may apply to different strains of HIV, including laboratory and primary isolates.

In fact, given the geographic diversity of distribution of different strains of HIV, it makes sense to utilize many strains of HIV with this methodology to make a comprehensive suite of immunogens and compositions thereof. The combined use of different strains of inactivated particles of HIV is what will confer to this potential vaccine preparation its polyvalent characteristics. Thus, in describing the method to generate immunogenic compositions and their testing for safety and efficacy, the inventor sought to establish the general principles of the methodology for its subsequent application. It should be understood that the inactivation of the HIV reverse transcriptase to be used in the initial study can be compared to other inactivators in parallel studies and thus allow for the selection of the most effective one.

It is of importance to note that the methodology of the present invention is applicable to any retrovirus which may be associated with any animal or human disease as a method for development of effective immunogens and preventive vaccines. Thus, the present invention has a broader applicability than the exemplified HIV vaccine.

4.1 HUMAN IMMUNODEFICIENCY VIRUS

The genetic diversity of HIV is due to the extremely high replication rate in infected individuals, the high rate of mutation caused by the error-prone reverse transcriptase, the substantial viral load, and selection within infected individuals (Doolittle, 1989; Ho *et al.*, 1995; Piatek *et al.*, 1993). Diversity is so great that the presence of closely related but not identical strains of HIV, known as quasispecies,

commonly appear in a single, infected individual. The quasispecies may diverge increasingly over time and changes tend to be within the *env* gene, particularly the V3 region (Hwang *et al.*, 1992). Although changes also may occur in the *gag*, *pol*, and accessory genes, these differences tend to be less substantial.

5

When significant changes accumulate and are seen in a large group of individuals, the strain is commonly considered a new family or new clade of HIV. Phylogenetic studies of HIV have shown that there are two major families of HIV, HIV-1 and HIV-2. Within the HIV-1 family there are two major antigenic groups, known as Group M (major) and Group O (outlier). Each of these two groups has in turn different subtypes or clades which, when analyzed, lead to the conclusion that both probably originated from two primordial viral ancestors. The group M is responsible for most of the HIV infections throughout the world and the group O is rarely found and confined to Cameroon, Gabon and France. There are at least nine subtypes or clades in the group M and of these, the subtype B is prevalent in the Western Hemisphere, while the subtypes A, C and D are in Africa. In Asia, the most frequently found subtypes are E, C and B, with the subtype E having a high prevalence in Southeast Asia. In India the prevalent subtype is C. A phylogenetic tree based on the sequence data from the *env* gene C2V2 region of selected isolates of the different subtypes is shown in FIG. 1. The geographic distribution of the different clades is shown in Table 1 (Hardy, 1996).

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TABLE 1
Worldwide Geographic Distribution of HIV-1 Subtypes and HIV-2

	HIV-1 Subtypes											HIV-2
	Group M											
	A	B	C	D	E	F	G	H	I	O		
Africa	+	+	+	+	+	+	+	+		+	+	
Middle East									+			
Europe	+	+	+	+		+	+	+		+	+	
Asia		+	+		+							
India	+	+	+								+	
Australia		+										
North America		+									+	
South America		+	+		+							

A vaccine comprising one clade may provide for the protection of infection by one or more other clades. A very important concept when confronting what appears to be the very difficult challenge of antigenic variation is the understanding of the concept of critical antigenic consistency. By critical antigenic consistency it is meant that there is a critical number of epitopes which are found consistently in HIV. Although it is recognized that there are significant antigenic changes in the configuration of the envelope proteins, generally, the internal proteins have less sequence variation. It has been recently demonstrated that epitopes, of critical immunologic importance, are exposed or created as HIV begins to fuse with cell membranes. The fusion process results in a conformational change of envelope glycoproteins leading to the exposure of previously occult epitopes or the de novo formation of epitopes. The recent use of these fusion exposed epitopes has led to the preparation of antibodies which are capable of inhibiting the infectivity of multiple primary HIV isolates, including multiple genetic subtypes (Montefiori and Moore, 1999; LaCasse *et al.*, 1999). The broad immunological protection elicited by the fusion exposed epitopes may explain the observation that people infected with HIV-1 virtually never have more than one subtype of virus.

These significant recent results indicate that once the immune system is exposed to HIV without integration of HIV in the genetic machinery of the host, the immune response will be effective and of a broad base. The non-infectious HIV particles of the part invention mimic the antigenic structure and composition of natural infectious HIV particles. Thus, these non-infectious particles will penetrate susceptible cells, including cells of the immune system responsible for the generation of the immune response, in the identical fashion as infectious particles, that is by receptor/co-receptor binding and fusion. The receptor-mediated entry of the vaccine into cells will result in exposure of the superior immunogenic epitopes and thereby facilitate the creation of a broad immunogenic response.

In addition to the recently described fusion exposed epitopes, the consistent regions of the *env*, *gag*, and *pol* together can lead to a critical mass of antigens responsible for the production of an effective immunological response to HIV and, which in fact, are present in nearly all types and subtypes of HIV. Thus, although it will be wise to use different wild types to create non-infectious particles and create a polyvalent vaccine, it is also possible that exposure of the immune system to a single type of inactivated HIV particle will be enough to generate a broad immune response.

The antigenic configuration of HIV is of the utmost importance since it is known that conformational epitopes can be located in variable regions of the HIV particle and can not be predicted from the analysis of the linear sequences of these regions. Therefore, it is of great importance that, in eliciting an effective protective immune response against HIV, the immune system is presented correct antigenic conformations.

Substantial evidence indicates that dendritic cells ("DC") present in epithelial tissues (e.g., Langerhans cells) are the initial cells infected with HIV after mucosal exposure to the virus (Cameron *et al.*, 1996; Knight, 1996). The bone marrow-derived DC are a class of antigen-presenting cells ("APC") that survey epithelial tissues for antigens and are efficient stimulators of both B and T lymphocytes. Unlike B cells, T

cells cannot directly recognize antigens and require that antigens be processed and presented by APCs (Banchereau and Steinman, 1998). Intracellular processing of antigens to peptide fragments results in binding to MHC class I molecules and a CD8+ cytotoxic T cell response. In contrast, antigens that enter DC by the endocytic pathway
5 generally bind to MHC class II molecules the elicitation of a CD4+ helper T cell response (Banchereau and Steinman, 1998).

Inactivated HIV viral particles will be processed and presented by DC as long as the inactivated HIV particles are preserved in its antigenic composition and can access the
10 cytoplasm of the dendritic cells. Both of these conditions are met by the present invention. That is, the inactivated particles have a preserved envelope structure and thus will access the cytoplasm of the dendritic cell by a process of micropinocytosis or mannose-receptor mediated uptake. DC that have been exposed to the inactivated HIV particles will migrate to the lymph nodes where they will interact with T-cells presenting
15 MHC-antigens complexes to both memory and naive T-cells (see Banchereau and Steinman, 1998; Bender *et al.*, 1995). This process will lead to the development of an effective anti-HIV MHC-I restricted CD8+ T-cell response. Cytotoxic CD8+ T cells are recognized as having an important role in controlling HIV invention (Musey *et al.*, 1997; Oldstone, 1997).

20 Dendritic cells also have CD4/HIV co-receptors and thus can be infected by HIV. This infectious process is independent of the capture and processing of HIV for antigenic presentation and initiation of the MHC class I restricted immunological response (Blauvelt *et al.*, 1997). But since the inactivated particles are non-infections, the process
25 of penetration through a receptor mechanism will allow the production of a MHC-II restricted response. Thus DC cells will activate and expand CD4+ T helper cells, which in turn will induce B cell growth and antibody production. This MHC class II response will thereby complement the MHC class I restricted immune response by establishing an effective cytotoxic and humoral response as well as an effective immunological memory.

The inventor contemplates that the present invention may be comprised of inactivated viruses from one or more clades of HIV. In preferred embodiments, the inactivated viruses may be comprised of inactivated viruses of the clade or clades which with an individual is most likely to come in contact. The data of Table 1 may be used as
5 a guide to determine which clades are prevalent in different geographical areas.

Because the present invention may be produced cheaply and rapidly, an individual may be vaccinated with inactivated virus or even inactivated HIV infected cells from the individual most likely to pass or have passed the virus to the individual. For example, an
10 HIV-negative person may be vaccinated with inactivated HIV or inactivated HIV-infected cells from an HIV-positive individual with which the HIV-negative individual plans to or has already come in sexual contact. An example of such a HIV-negative individual could be someone married to a hemophiliac that is HIV-positive. Additionally, these "personal" vaccines may have the benefit of also having cellular
15 (nonviral) surface proteins from the individual passing the virus. The immune response to cellular surface proteins incorporated into the virus particles, which include MHC antigens, have been shown to confer protection from future challenges from viruses grown in the same cell line (Stott *et al.*, 1991).

20 4.2 PHOTOINACTIVATION OF REVERSE TRANSCRIPTASE

A number of non-nucleoside inhibitors of HIV reverse transcriptase have been described and include nevirapine and its analogs, the pyridobenzo- and dipyrroldiazepinones, the pyridones, the quinoxalines, and the carboxanilides. Specific compounds include 9-AN, UC781™, UC38, UC84, UC10, UC82, UC040, HBY 097,
25 calanolide A, U-88204E, and many others (Barnard *et al.*, 1997; Esnouf *et al.*, 1997; Buckheit *et al.*, 1997; Kleim *et al.*, 1997; Currens *et al.*, 1996; Althaus *et al.*, 1993). These compounds may be converted to azido photoaffinity labels and utilized for the inactivation of HIV particles using methods described herein. The inventor contemplates that essentially any compound that binds and inhibits HIV RT, is able to penetrate the
30 viral particle and associate with RT, and does not cause significant alterations in the

conformation of the virus particle may be used to produce an RT-inactivated virus for the purpose of eliciting a protective immune response in an individual. Furthermore, the inventor contemplates that the exposure of the photaffinity label-treated particles to light radiation to irreversibly inactivate the RT may comprise of light of a variety of wavelengths. Although UV light, particularly that emitted by a GE 275 W sun lamp, is preferred, any exposure to light that causes the reaction of the azido compound with RT is contemplated to be of utility in the production of the compositions of the present invention.

10 4.3 VACCINE PREPARATION

The inactivation of the virus by photoinactivation of RT provides noninfectious, immunogenic particles that are essential identical in conformation and composition as infectious particles. Therefore, the inventor contemplates that particles inactivated in this method are ideal for use as a potential vaccine against HIV diseases including AIDS and
15 AIDS-related conditions. Thus the present invention provides an immunogenic composition that may be used as a vaccine against HIV infection and its consequences, including AIDS and AIDS-related conditions. The immunogenic composition may also be used to generate diagnostic antibodies, HIV-binding compounds, and diagnostic kits useful in the development of vaccines. The immunogenic compositions elicit an immune
20 response which produces cellular and humoral immune responses that are antiviral. A vaccinated host can be the source of diagnostic antibodies. If a vaccinated host is challenged by HIV, T cells of the cellular response will eliminate infected cells and antibodies of the humoral response will inactivate the virus by binding to its surface.

25 Vaccines may be injectable liquid solutions or emulsions. The RT-inactivated HIV particles may be mixed with pharmaceutically-acceptable excipients which are compatible with the inactivated virus particles. By compatible it is meant that the pharmaceutically-acceptable excipients will not alter the conformational characteristics of the viral particle. Excipients may include water, saline, dextrose, glycerol, ethanol, or
30 combinations thereof. The vaccine may further contain auxiliary substances, such as

wetting or emulsifying agents, buffering agents, or adjuvants to enhance the effectiveness of the vaccines. Adjuvants may be mineral salts (e.g., $\text{AlK}(\text{SO}_4)_2$, $\text{AlNa}(\text{SO}_4)_2$, $\text{AlNH}_4(\text{SO}_4)$, silica, alum, $\text{Al}(\text{OH})_3$, $\text{Ca}_3(\text{PO}_4)_2$, kaolin, or carbon), polynucleotides (e.g., poly IC or poly AU acids), and certain natural substances (e.g., wax D from *Mycobacterium tuberculosis*, substances found in *Corynebacterium parvum*, *Bordetella pertussis*, or members of the genus *Brucella*) (PCT Application No. 91/09603). Aluminum hydroxide or phosphate (alum) are commonly used at 0.05 to 0.1 percent solution in phosphate buffered saline. Other adjuvant compounds include QS21 or incomplete Freund's adjuvant.

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Vaccines may be administered parenterally, by injection subcutaneously or intramuscularly, or the vaccines may be formulated and delivered to evoke an immune response at the mucosal surfaces. The immunogenic composition may be administered to a mucosal surface by the nasal, oral, vaginal, or anal routes. The inventor contemplates that the administration of the immunogenic compound to a mucosal surface that is most likely to be challenged by HIV, such as the anal, vaginal, or oral mucosa, is preferred. For vaginal or anal delivery, suppositories may be used. Suppositories may comprise binders and carriers such as polyalkylene glycols or triglycerides. Oral formulations may be in the form of pills, capsules, suspensions, tablets, or powders and include pharmaceutical grades of saccharine, cellulose or magnesium carbonate. These compositions may contain 10% to 95% of the RT-inactivated viral particles.

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Preferably the vaccines are administered in a manner and amount as to be therapeutically effective. That is to say that the vaccine should be administered in such a way as to elicit an immune response to the RT-inactivated viral particles. Suitable doses required to be administered are readily discernible by those of skill in the art. Suitable methodologies for the initial administration and booster doses, if necessary, may be variable also. The dosage of the vaccine may depend on the route of administration and may vary according to the size of the host. One of skill in the art may obtain details regarding the practice and use of the present invention in the American Foundation for

AIDS Research's HIV Experimental Vaccine Directory, Vol 1, No. 2, June 1998, which is hereby incorporated by reference in its entirety.

Although the immunogenic compositions of the present invention may be administered to individuals that are not infected with HIV, HIV-negative, they also may be administered to individuals who are infected with the virus in an effort to alter the immune response to the virus. The alteration may be a stimulation of anti-HIV CD4⁺ or CD8⁺ T cells, an increase in antibody production, or in respect to the type of response to the virus (*i.e.*, T_H1 vs. T_H2). Nonetheless, this alteration if effective will decrease the mortality and morbidity associated with the HIV infection. In other words, the immunogenic compound may decrease the severity of the disease and increase the life of the patient.

4.4 PHARMACEUTICAL COMPOSITIONS

Where clinical application of an immunogen according to the present invention is contemplated, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the inactivated virus, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of

such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

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Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The inactivated viruses and inactivated virus-producing cells of the present invention may include classic pharmaceutical preparations. Administration of pharmaceutical compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal, intraocular, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients.

The pharmaceutical compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives,

5 buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to well known parameters.

10 The compositions of the present invention may comprise a supplement of one or more compounds capable of preventing the replication of HIV, including the compound utilized to inactivate the virus. These compounds may include, but are not limited to, nucleoside analog inhibitors of HIV RT (*e.g.*, AZT), non-nucleoside inhibitors of HIV-RT (*e.g.*, UC781™), or HIV protease inhibitors.

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4.5 SAFETY OF THE IMMUNOGENS

The safety of the immunogens may be demonstrated by their inability to produce infection in susceptible cells regardless of the amount of particles used as inoculum. Controlled studies may be conducted exposing susceptible cells to increased concentrations of these particles. Particles which have their RT inactivated will fail to infect susceptible cells, while the control studies will maintain the capacity to produce infection in the susceptible cells. The same methodology that was used to generate the viral particles may be used to test the inactivation of the virus particles of the present invention. For monitoring infectivity in both the non-infectious particles and the controls, the inventor contemplates the monitoring of production of RT and p24 antigen in the culture supernatants. In a preferred embodiment, supernatants are tested for the presence of virus particles by the sensitive method of heminested polymerase chain reaction (HNPCR) amplification of the 5' LTR sequences (LTR-HNPCR). This test will confirm the absence of infectivity of the particles since there is an excellent correlation between a negative infectivity test and a negative LTR-HNPCR (Yang *et al.*, 1998).

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The safety of the particles can also be evaluated *in vivo* by inoculation of the animal models discussed *infra* in section 4.6. The lack of infectivity of the inactivated particles can be determined by repeated high dose inoculation of animals such as PBL-SCID mice, SCID-hu mice, or non-human primates.

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As a way of creating an additional safety mechanism for the compositions of this invention, HIV integrase, an enzyme required for viral integration, can be inactivated. It is important to clarify that since the reverse transcriptase of the viral particle is inactivated there will be no replication of the virus. The inactivated of HIV integrase would be an added safety feature. Without a functional integrase there is no possibility for the integration of HIV into the genetic material of the cell further ensuring the safety of the vaccine. The mechanism for integrase inactivation will be one of selective photolabeling using a (as azido group) bound to any of several compounds that are known to bind to HIV-integrase. Among these compounds are: anti-integrase oligonucleotides, L-chicoric acid, as well as a large number hydrazine derivative inhibitors.

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4.6 ADMINISTRATION

Although it is important to consider different routes of administration, the intramuscular route will be the route of choice. Other routes include: 1) intranasal; 2) intrarectal; 3) intravaginal; 4) oral and 4) subcutaneous. The dose to be used will be measured in viral particles and it will have a range from the administration of 1 particle to 10^{20} particles. It is anticipated that the optimal range of dosing will be between 10^4 particles and 10^8 particles. Thus lower dose ranges may include doses of about 10 , 10^2 , or 10^3 particles. Optimal dose ranges may include doses of about 10^4 , 10^5 , 10^6 , 10^7 , or 10^8 particles. Higher dose ranges may include doses of about 10^{10} , 10^{12} , 10^{14} , 10^{16} , 10^{18} or 10^{20} particles. The effective dosage may vary depending on the method of administration.

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For each dose to be tested, the schedule may consist of administration of a dose on days 0, 30, 60, and a booster dose at 180 days. Alternatively doses may be given weekly, every two weeks, or monthly for periods of one, two, three, four, five or six

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months. Doses may also be given every two months for a similar time. Periodic booster shots at intervals of 1-5 years may be desirable to maintain protective levels of immunity or generate sufficient immune response. Other administration schedules may be used and the invention contemplates any administration schedule that results in an effective response.

In addition to monitoring for clinical safety, efficacy will be assessed by measuring the cellular and humoral immune response to HIV. Subjects will be followed for a period of two or more years from day 0 (date of first inoculation).

4.7 ANIMAL MODELS

A number of different animal model systems for HIV infection have been employed (Kindt *et al.*, 1992). Non-human primates such as chimpanzees and pig-tailed macaques can be infected by HIV-1. Although CD4+ cells are not depleted in these systems, the animals are detectably infected by the virus and are useful in determining the efficacy of HIV vaccines. Small animal models include chimeric models that involve the transplantation of human tissue into immunodeficient mice. One such system is the hu-PBL-SCID mouse developed by Mosier *et al.* (1988). Another is the SCID-hu mouse developed by McCune *et al.* (1988). Of the two mouse models, the SCID-hu mouse is typically preferred because HIV infection in these animals is more similar to that in humans. SCID-hu mice implanted with human intestine have been shown to be an *in vivo* model of mucosal transmission of HIV (Gibbons *et al.*, 1997). Methods of constructing mammals with human immune systems are described in U.S. Patents 5,652,373, 5,698,767, and 5,709,843.

The animals will be inoculated with the immunogens of the present invention and later challenged with a dose of infectious virus. Efficacy of the immunogens in producing a protective response will be determined by methods known by those of skill in the art. Generally, a variety of parameters associated with HIV infection may be tested and a comparison may be made between vaccinated and non-vaccinated animals. Such

parameters include viremia, detection of integrated HIV in blood cells, loss of CD4+ cells, production of HIV particles by PBMC, etc.. The immunogens will be considered effective if there is a significant reduction of signs of HIV infection in the vaccinated versus the non-vaccinated groups.

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The ability of the inactivated HIV particles to elicit neutralizing antibodies can be measured in mice as previously described (LaCasse *et al.*, 1999). The ability of sera to neutralize a range of HIV isolates can be tested using U87-CD4 cells expressing either CCR5 or CXCR4 coreceptors or by using an peripheral blood lymphocyte culture assay
10 (LaCasse *et al.*, 1999, LaCasse *et al.*, 1998; Follis *et al.*, 1998).

4.8 APPLICATION IN HUMANS

Of course, the inventor contemplates the application of the present invention as a vaccine to HIV in humans. The inventor contemplates that testing of the present
15 invention as a vaccine in humans will follow standard techniques and guidelines known by those of skill in the art. One important aspect of human application is the production of an effective immune response to the vaccine. Although various *ex vivo* tests may be performed, such as measuring anti-HIV antibody production and anti-HIV cellular responses, the ultimate test is the ability of the vaccine to prevent infection by HIV or to
20 significantly prolong the onset of AIDS in individuals receiving the vaccine. The monitoring of the efficacy of HIV vaccines in humans is well known to those of skill in the art and the inventor does not contemplate that the present invention would require the development of new methods of testing the efficacy of an HIV vaccine.

25 5.0 EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute
30 preferred modes for its practice. However, those of skill in the art should, in light of the

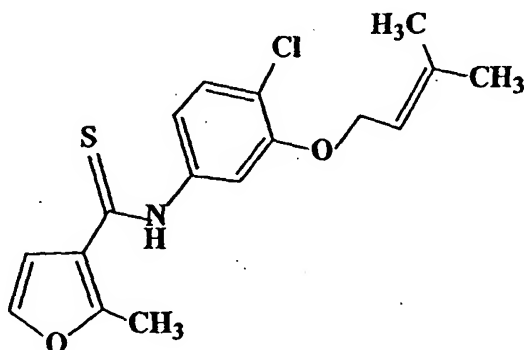
present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5 5.1 EXAMPLE 1 -- PHOTOAFFINITY LABELS

One compound that may be used to photoinactivate the reverse transcriptase of HIV-1, is an azido dipyridodiazepinone, 9-Azido-5,6-dihydro-11-ethyl-6-methyl-11H-pyrido[2,3-b][1,5]benzodiazepin-5-one (9-AN). Production of 9-AN is described in Hargrave *et al.* (1991). It may be prepared by mixing an equimolar mixture of 2-chloronicotinic acid and 4-nitrophenylendiamine heated in sulfolane at 170°C for 5
10 hours. After cooling, the precipitate is collected and washed with hot ethanol. The obtained mixture of 8- and 9- nitro-5,6-dihydro-11 H -pyrido[2,3-b]benzodizepin-5-ones, are then methylated with methyl iodide and dimsylsodium in DMSO, and the 5,6-dihydro-6-methyl-9nitro isomer is purified by fractional crystallization. The 8-amino
15 compound is obtained by ethylation with ethyl iodide and dimsylsodium in DMSO followed by stannous chloride reduction of the nitro group. The 8-amino compound is then converted to the azide by diazotization with sodium nitrate followed by reaction with sodium azide. The 9-azido derivative is then crystallized from diethyl ether to give analytically pure material: mp 115-118°C; CIMS molecular ion H⁺ 295; C,H, and N
20 analysis and spectroscopic characterization were consistent with the structure. A methanolic solution of the azido photolabel has a lambda max of 248 nm($\epsilon=30\ 000$).

Another molecule that can be prepared as a photolabel by diazotization is a thiocarboxanilide, known by the trademark name of UC781TM. UC781TM was developed
25 by Uniroyal Chemical Ltd. Research laboratories. It is known that UC781TM is a molecule with high affinity for HIV-1 reverse transcriptase and with the characteristics of tight binding. In fact, it is reasonable to assume that UC781TM alone inactivates the HIV-1 reverse transcriptase (Barnard *et al.*, 1997; Borkow *et al.*, 1997). The diazotization of this molecule and its subsequent activation with ultraviolet light further insures the
30 inactivation of the HIV-1 reverse transcriptase. The azido photoaffinity labels, upon

exposure to ultraviolet light, transform into highly reactive nitrenes capable of inserting into proximal covalent bonds of the HIV-1 reverse transcriptase enzyme structure.



Structure of UC781™

5.2 EXAMPLE 2 — INACTIVATION OF HIV PARTICLES AND HIV-INFECTED CELLS

The inactivation of HIV can be accomplished by taking advantage of compounds that bind the HIV reverse transcriptase with a high degree of specificity. Using the technique of photolabeling, a compound with high specific affinity for the HIV-1 reverse transcriptase can then be turned into an "active" moiety which will produce an irreversible inactivation of the reverse transcriptase upon exposure to ultraviolet light irradiation. This inactivation, in the case of the compounds already described and known to have this effect, meets the requirement of being specific for the reverse transcriptase of HIV-1. That is, their labeling and photoactivation is not accompanied by the alteration of any other component of the viral particle than the reverse transcriptase of HIV-1. That is an important element of this invention since the irreversible inactivation of HIV-1 reverse transcriptase will lead to non-infectious particles of HIV-1 with a natural antigenic structure which should behave as the infectious particles of HIV-1 insofar as their capacity for stimulation of an effective immune response. There are many compounds that can be used for the purpose of photolabeling inactivation of the HIV-1 reverse transcriptase and include UC781™ thiocarboxanilide, UC781™ azidothiothiocarboxanilide, and azido dipyrroldiazepinona.

5.2.1 INFECTION OF VIRUS PRODUCING CELLS

Laboratory-adapted and primary wild type HIV isolates will be cultured using established standard techniques. The cells to be used for culture will be phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC's), since they are more readily infected by both primary isolates and laboratory-adapted isolates than cloned T-cell lines (MT-2 or H9). Briefly, PBMC from normal blood donors are isolated by Ficoll-Hystopaque gradient centrifugation and stimulated with 5 µg/ml of PHA for three to four days. The PHA-stimulated PBMC are then cultured in RPMI-1640 medium containing 10% heat-inactivated FBS, 100 U penicillin/ml, 100 µg of streptomycin/ml 2 mM L-glutamine, and 5% purified human interleukin-2.

Aliquots of 10^7 uninfected PBMC's in 10 ml of medium are pretreated with 2 µg/ml of polybrene for 1 h at 37°C. The cells are then infected with a 1.0 ml inoculum of cell-free supernatant of the primary isolate or the laboratory-adapted isolate. The infected PBMC's are then resuspended in the culture media and monitored for supernatant p24 antigen concentration, which generally peaks at day 14 postinoculation. The culture supernatants are harvested, pooled, and clarified through a 0.45 µm filter, and aliquoted. The 50% tissue culture infectious dose is determined according to the protocol described by Johnson *et al.*, (1990) using the HIV p24 antigen detection technique.

Of course, the inventor contemplates that the use of PBMCs may not be feasible when large volumes of virus are needed. In this instance, the cell line utilized is MT-2 grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FBS), glutamine and antibiotics. Cells are propagated at 37°C in an atmosphere of 5% CO₂ in air. The virus employed for this work is HIV-1 isolates IIIB and/or RF, which are prepared by an acute infection process. Virus infection of the MT-2 cells is carried out in a bulk infection process. The appropriate number of cells is mixed with infectious virus in a conical centrifuge tube in a small total volume of 1-2 milliliters. Following a 4-hour incubation, the infected cells are brought to the appropriate final concentration of 5×10^4

cells per milliliter with fresh tissue culture medium. Uninfected cells at the same concentration are plated for the toxicity controls and for the cell controls. The MOI is adjusted to give complete cell killing in the virus control wells by Day 6. Virus particles are concentrated using standard techniques and quantified using RT assays (Fletcher *et al.*, 1995a, 1995b) and p24 antigen assays.

5.2.2 HIV PARTICLE INACTIVATION

Once the HIV particles are purified and quantified, the 50% tissue culture infective dose ($[TCID_{50}] = 5 \times 10^4$) is incubated in the presence of four to eight times the 50% inhibitory concentration (IC_{50}) of the photoaffinity labeling molecule. In the case of the azido dipyrroldiazepinone, the IC_{50} is 160 nM. For the thiocarboxanilide and the azido thiocarboxanilide, the IC_{50} is 0.2 nM. Incubations were in RPMI 1640 without FBS for 2 h at 37°C with gentle agitation every 15 min. The mixtures of viral particles and photoaffinity labels are exposed to ultraviolet light using a GE 275-W sun lamp that provides a UV-irradiation intensity of about $15 \mu W/cm^2$ for a period of at least 50 minutes. After this process, the solution contains particles of HIV-1 with a completely inactivated reverse transcriptase and thus unable to infect susceptible cells.

The infectivity of the inactivated virus particles will be determined by controlled experiments where exposure of susceptible cells to increased concentrations of these particles will fail to produce infection of the cells as evidenced by the sensitive heminested PCR technique described in Yang *et al.* (1998) and the lack of production of viral particles, reverse transcriptase, and p24. Such a process to assay the production of virus particles is outlined by Borkow *et al.* (1997). Briefly, 0.5 ml of concentrated inactivated virus is added to 0.5 ml of phytohemagglutinin-activated cord-blood mononuclear cells (CBMC) (4×10^6 cells) in RPMI 1640-10% FBS and incubated for 2 h at 37°C with occasional gentle mixing. The HIV-CBMC incubation mix is diluted with the addition of 10 ml of RPMI 1640, and residual HIV is removed by pelleting the cells at $300 \times g$ for 10 min, followed by removal of the supernatant and resuspension of the cells in 2 ml of RPMI 1640-10% FBS containing interleukin-2 (10 U/ml). The entire sample is

plated into a single well of a 24-well dish. After 4 days of culture, 1 ml of medium is removed and replaced with 1 ml of fresh medium. On day 7, culture supernatants are isolated and HIV production is assessed by the measurement of RT activity and p24 antigen levels in these cell-free supernatants and cells are monitored for integration by the heminested PCR technique of Yang *et al.* (1998).

5.2.3 MANIPULATION AND PREPARATION OF HIV PARTICLES USING PHOTOINACTIVATED VIRUS

HIV isolates, whether cultured in laboratory conditions, or isolated from hosts are more safely handled and analyzed after photoinactivation. Inactivation may be accomplished in samples of host tissues by providing sufficient amounts of RT-binding compounds. Host samples may include, but are not limited to, blood, sera, semen, vaginal tissues, anal tissues, nervous and muscle tissues, saliva, tears, and other bodily fluids. HIV types that may be present in these samples include, but are not limited to, HIV 1, HIV 2, Groups M, O, or I, and Subtypes A through H.

The samples will be incubated in the presence of four to eight times the 50% inhibitory concentration (IC₅₀) of the photoaffinity labeling molecule. In the case of the azido dipyrindiazepinone, the IC₅₀ is 160 nM. For the thiocarboxanilide and the azido thiocarboxanilide, the IC₅₀ is 0.2 nM. Incubations of tissues may be in RPMI 1640 without FBS or other, suitable media. Incubations may last about one minute, 15 minutes, 30 minutes, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, or 24 hours or more, depending upon the permeability of the tissue to the RT binding compound. The mixtures of samples and photoaffinity labels are exposed to ultraviolet light at an intensity and duration sufficient to photoactivate the binding of the RT binding compound and inactivate RT. For example, for some samples, a GE 275-W sun lamp, which provides a UV-irradiation intensity of about 15 μW/cm² may be used for a period of at least 50 minutes. After this process, the mixture contains particles of HIV-1 with a completely inactivated reverse transcriptase and thus unable to infect susceptible cells.

Additionally, viral particles may be isolated prior to inactivation, inactivated, then more safely stored or analyzed. Viral particles are isolated from infected tissues, tissue or cell cultures, or other source through techniques well known in the art. Such isolation may be through cell disruption followed by density gradient centrifugation. The purified virus can be any retrovirus of interest, including, but not limited to, HIV 1, HIV 2, Group M, I, or O, and Subtypes A through H. Analysis of the components and properties of such viruses may include, but are not limited to those as disclosed in U.S. Patent 6,017,543, incorporated herein by reference.

5.3 EXAMPLE 3 -- PRODUCTION OF NONINFECTIOUS NASCENT VIRUS FROM UC781TM-TREATED HIV INFECTED CELLS

In addition to the inactivation of purified virus, UC781TM has been shown to inactivate nascent virus from HIV-infected cells grown in the presence of the compound (Borkow *et al.*, 1997, incorporated wherein by reference). The methods described by Borkow *et al.* may be used to produce inactivated virus for use in the present invention. Furthermore, the methods may be used to create a whole-cell vaccine in which the cells are HIV infected but rendered noninfectious by UC781TM. A whole cell vaccine comprises the injection of HIV-infected cells. The injection of whole cells may provide a more vigorous immune response to the virus. The methods of Borkow *et al.* (1997) are described below.

5.3.1 INCUBATION OF CHRONICALLY HIV-1 INFECTED H9 CELLS WITH UC781TM

Chronically infected H9 cells (5×10^5 cells) are incubated with 10 μ M of UC781TM in a total volume of 1 ml of RPMI 1640-10% FBS for 18 h at 37°C. The cells are then separated from the culture supernatants by centrifugation at $300 \times g$ for 10 min. The pelleted H9 cells are washed by suspension in 10 ml of RPMI 1640 followed by centrifugation at $300 \times g$ for 10 min. The cell pellet is resuspended in 4 ml of RPMI 1640-10% FBS and used in coculture experiments to determine infectivity (Borkow *et al.*, 1997).

5.3.2 INCUBATION OF PERIPHERAL BLOOD LYMPHOCYTES WITH UC781™

Peripheral blood lymphocyte (PBL) cells (2×10^6 cells) isolated from blood of HIV-1-infected patients are incubated with medium and 10 μ M UC781™ in a total volume of 1 ml for 2 h at 37°C. Excess drug may be removed by pelleting the cells by centrifugation at $300 \times g$ for 10 min and removal of the medium. The cell pellet is washed by suspension in 10 ml of medium followed by centrifugation. This washing step is repeated twice. The final cell pellet is resuspended in 1 ml of medium or another isotonic solution. To insure that the cells are noninfectious, they are cocultured with 1 ml of activated CBMC (2×10^6 cells). The culture medium is changed every 2 days, and fresh activated CBMC (2×10^6 cells) are added once per week. HIV-1 production is monitored by measurement of p24 antigen levels in cell-free culture supernatants. Integration of the virus is tested by the heminested PCR technique of Yang *et al.* (1998).

5.4 EXAMPLE 4 – CLINICAL TRIAL FOR HIV VACCINE

This example describes a protocol to facilitate an HIV vaccine clinical trial. The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. Generally, the clinical study of the vaccine composed of inactivated viral particles should consist of the administration of such viral particles produced by the photolabeling of reverse transcriptase, as described in the present invention, to human subjects to evaluate safety and cellular, antibody, humoral and other clinical responses. The following information is being presented as a general guideline for use in HIV vaccine clinical trials. Information regarding design of clinical trials can also be obtained in the American Foundation for AIDS Research's HIV Experimental Vaccine Directory, Vol 1, No. 2, June 1998.

5.4.1 Eligible Subjects

Adult males and females HIV seronegatives.

5.4.2 Subjects Inclusion Criteria

Patient Age: 18 years - 60 years.

5.4.3 Reproductive Criteria

- 5 Negative pregnancy test. Abstinence or effective method of birth control/contraception during the study.

5.4.4 Inclusion Criteria

- 10 The subject must be healthy as defined by a normal physical exam and normal laboratory parameters as defined by the WHO for participants in clinical studies. Subjects must be able to understand and sign an informed consent. Subjects must also have a normal total white blood cell count, lymphocyte, granulocyte and platelet count as well hemoglobin and hematocrit. Subjects must have normal values of the following parameters: urinalysis; BUN; creatinine; bilirubin; SGOT; SGPT; alkaline phosphatase; 15 calcium; glucose; CPK; CD4+ cell count; and normal serum immunoglobulin profile.

5.4.5 Exclusion

- 20 The following are exclusion criteria: HIV-seropositive status; Active drug or alcohol abuse; inability to give an informed consent; medication which may affect immune function with the exception of low dose of nonprescription-strength NSAIDS, aspirin, or acetaminophen for acute conditions such as headache or trauma; any condition which in the opinion of the principal investigator, might interfere with completion of the study or evaluation of the results.

25 5.4.6 Randomization

The study will be double blind randomized. The placebo will be the vaccine solution without the inactivated viral particles. Subjects will be assigned randomly to one of the vaccine routes described above.

5.4.7 Dose range

Doses of 10^4 , 10^6 and 10^8 particles will be studied for clinical safety and immunogenicity. Other doses in the range of 10 to 10^{20} particles may also be studied.

5 5.4.8 Administration

For each dose to be tested, the schedule may consist of administration of a dose on days 0, 30, 60, and a booster dose at 180 days. Route of administration will be intramuscular. Additional routes of administration may include: subcutaneous; oral; intrarectal; intravaginal; intranasal/intramuscular; intrarectal/intramuscular; 10 intranasal/subcutaneous; intrarectal/subcutaneous

5.4.9 Number of subjects per route of administration

There will be 12 subjects per route of administration per dose level. Of the 12 subjects 8 will receive the vaccine and 4 will receive a solution without inactivated viral 15 particles.

5.4.10 Duration of the study

24 months.

20 5.4.11 Endpoints

The endpoint for clinical safety is no evidence of alteration of the clinical, immunological or laboratory parameters. The endpoint for immunological efficacy is seroconversion with production of an effective cellular, humoral and antibody response against HIV. The effective immunological cellular response can be studied by using 25 cytotoxic T lymphocytes responses against different clades of HIV. The humoral response can be evaluated by measuring the production of IFN-gamma release using a modified Elispot assay. The antibody production can be assessed by performing neutralization studies against different clades of HIV.

5.5 EXAMPLE 5 — IMMUNOGEN PREPARATION

The immunogenicity of whole, inactive HIV particles is well known in the art. In particular, U.S. Patent 5,849,475 discloses effective immunogenicity of intact, inactive HIV virus particles, and is incorporated herein by reference.

5

Inactive viral particles produced as described above in sections 5.2.1 and 5.2.2 are tested for inactivity by the methods of section 5.2.2. The inactivated viral particles are concentrated and purified by standard procedures involving pelleting and sucrose gradient centrifugaion. The concentrated, purified virus is diluted to about 200 µg/ml in saline.

10 This aqueous phase may be added to an oil phase containing 9 parts mineral oil (Drakeol-6) and 1 part Arlacel A (mannide monooleate). This mixture is stored in a volume sufficient to provide for an equal volume of air and shaken on a Spex Model 8000 Mixermill to form a stable emulsion. Such an emulsion is expected to form after about 10 minutes of shaking and may be stored unfrozen at 4°C.

15

To demonstrate immunogenicity, vertebrate hosts are immunized with about 1 µg to about 1000 µg of p24-equivalent amounts of HIV particles, depending upon the size and species of the host. The particles may be adjuvanted in Freund's complete adjuvant. For guinea pigs, the amount of immunogen used is about 10 µg of p24-equivalent
20 amounts of HIV particles. The inoculated animals are typically boosted every three weeks for a total of three booster shots. Two weeks after the last shot, immune sera may be collected and assayed by ELISA for antigen reactivity. Antigen reactivity indicates successful immunogenic response.

5.6 EXAMPLE 6 -- IMMUNOASSAYS

This example describes the use of photoinactivated virus particles as antigens in immunoassays, non-enzyme linked antibody binding assays, or procedures known in the art for detection and measurement of anti-retroviral antibodies and retroviral antigens, e.g. HIV. Manipulation and uses of immunogens are well known in the art. See Harlow & Lane, *Antibodies: A Laboratory Manual*, incorporated herein by reference. Further procedures and methods of immunoassays and related techniques utilizing inactive or modified HIV virus particles can be found in the art, particularly in U.S. Patents 6,080,408, and 5,919,458 which are incorporated herein by reference.

An ELISA assay may be accomplished using inactive viral particles produced as described above in sections 5.2.1 and 5.2.2. In some embodiments of the ELISA assay, photoinactivated viral particles are immobilized onto a selected surface, preferably a surface exhibiting a protein affinity such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed material, one will desire to bind or coat a nonspecific protein such as bovine serum albumin (BSA), casein, solutions of milk powder, gelatin, PVP, superblock, or horse albumin onto the well that is known to be antigenically neutral with regard to the test antisera. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface. Following an appropriate coating period (for example, 3 hours), the coated wells will be blocked with a suitable protein, such as bovine serum albumin (BSA), casein, solutions of milk powder, gelatin, PVP, superblock, or horse albumin, and rinsed several times (e.g., 4 or 5 times) with a suitable buffer, such as PBS. The wells of the plates may then be allowed to dry, or may instead be used while they are still wet.

After binding of antigenic material to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the antisera or clinical or biological extract to be tested in a manner conducive to immune complex (antigen/antibody) formation. Such

conditions preferably include diluting the antisera with diluents such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

5 In each of the microtiter wells will be placed about 10 µl of the test patient sample along with about 90 µl of reaction buffer (e.g., PBS, which may also contain about 1% digitonin or other mild protein solubilizing agent). Control wells of the ELISA plate may include normal sera (human sera without anti-HIV antibody) and anti-HIV antibody obtained from patients that have seroconverted using conventional anti-HIV antibody
10 detection techniques.

 The layered antisera is then allowed to incubate for from 1 to 4 hours, at temperatures preferably on the order of 20° to 25° C. Following incubation, the antisera-contacted surface is washed so as to remove non-immunocomplexed material. A
15 preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer.

 Following formation of specific immunocomplexes between the test sample and the bound particle, and subsequent washing, the occurrence and even amount of
20 immunocomplex formation may be determined by subjecting same to a second antibody having specificity for the first. Of course, in that the test sample will typically be of human origin, the second antibody will preferably be an antibody having specificity in general for human IgG, IgM or IgA. To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon
25 incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antisera-bound surface with a urease, alkaline phosphatase, or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

30

After incubation with the second enzyme-tagged antibody, and subsequent to washing to remove unbound material, the amount of label is quantified by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid [ABTS] and H_2O_2 , in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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U.S. Patent 5,516,629.

U.S. Patent 5,593,823.

U.S. Patent 5,652,373.

U.S. Patent 5,698,767.

U.S. Patent 5,709,843.

U.S. Patent 5,849,475.

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